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Synthesis, biological activity, and conformational analysis of CD-ring modified *trans*-decalin 1α,25-dihydroxyvitamin D analogs †

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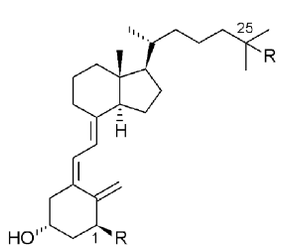
Received 18th September 2002, Accepted 2nd December 2002
First published as an Advance Article on the web 19th December 2002

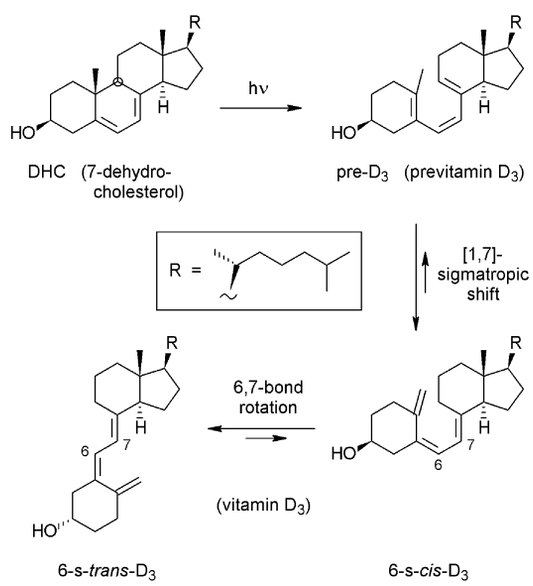
A novel series of analogs of 1,25-dihydroxyvitamin D_3, the hormonally active metabolite of vitamin D_3, characterised by the presence of a *trans*-fused decalin CD-ring system, possesses surprising biological activities in combination with specific structural modifications in the flexible parts of the molecule, when compared with the natural hydrindane derivatives. (1) A large difference in biological activity is observed between the 20-epimeric *trans*-decalin analogs that follows a pattern opposite to what is usually observed for the natural ring size. (2) Several *trans*-decalin analogs that are modified in the seco-B-ring region, including previtamin derivatives, possess a pronounced vitamin D-like activity, whereas the corresponding hydrindane derivatives are inactive. The molecular origin of this behavior is still under study.

Introduction

For chemists, biochemists, medicinal chemists, and physicians alike, vitamin D represents a fascinating molecule.[1] Its chemical history started in the 1930's when Windaus proposed a surprising secosteroid structure with an intact cholesterol side chain for the substance,[2] a deficiency in which was known to be responsible for bone diseases such as endemic rickets and osteomalacia. Since the importance of calcium in bone formation was known at that time, vitamin D_3 was also called cholecalciferol (D_3). Many years later, it was discovered, in particular through the seminal work of DeLuca, Kodicek, and Norman that the biological action of the vitamin originates from the dihydroxylated metabolite 1α,25-dihydroxyvitamin D_3 (1,25-D_3), also called calcitriol, which functions as a hormone.[3–5] As was subsequently assessed, these actions not only involve regulation of calcium homeostasis, but also immunomodulation, selected cell differentiation, and antiproliferation.[6] At the molecular level 1,25-D_3 stimulates biological responses *via* signal transduction pathways that utilize either a nuclear receptor (n-VDR) for regulating gene transcription (slow genomic pathway) or a putative membrane receptor (m-VDR) for its rapid actions such as transcaltachia (non-genomic pathway).[7]



D_3 R = H (cholecalciferol)
1,25-D_3 R = OH (calcitriol)



Scheme 1 Biosynthetic pathway to vitamin D.

The characteristic seco-structure of vitamin D originates from the photolytic cleavage of the C-9,C-10 bond upon UV irradiation of the provitamin 7-dehydrocholesterol (Scheme 1). The so generated previtamin triene (pre-D_3) then undergoes a reversible thermal [1,7]-sigmatropic hydrogen shift leading to the vitamin form in its less stable s-*cis* conformation. Rapid rotation around the C-6,C-7 single bond eventually yields D_3 in its more stable extended conformation.[8,9] It is believed now that the transoid and cisoid conformations represent the active geometries of the hormone when binding to the n-VDR and m-VDR, respectively.[10] In this context the previtamin form is unique in that it corresponds to a vitamin tautomer whose geometry is locked in the cisoid form.

The vast therapeutic potential of 1,25-D_3 that is associated with the inhibition of cell growth and with the stimulation of cell differentiation is however hampered by the observation that

† Electronic supplementary information (ESI) available: Further experimental details. See http://www.rsc.org/suppdata/ob/b2/b209147j/

DOI: 10.1039/b209147j

above the required physiological doses the hormonally active metabolite leads to calcemic side effects such as hypercalcemia, hypercalciuria and bone decalcification. This has originated in a search for structural analogs of 1,25-D_3 that show a separation in calcemic and antiproliferative–prodifferentiating activities. A turning point in this research constitutes the recent disclosure by Moras of the detailed structure of the ligand binding domain (LBD) of the n-VDR, obtained *via* X-ray diffraction analysis of the complex between 1,25-D_3 and a truncated mutant of the n-VDR.[11]

In the context of analog development it is appropriate to distinguish three different parts in the structure of 1,25-D_3: a central rigid CD-bicyclic ring portion to which are connected, at C-17 and at C-8, two flexible entities, the side chain and the seco-B,A-ring system, respectively (Chart 1). Although

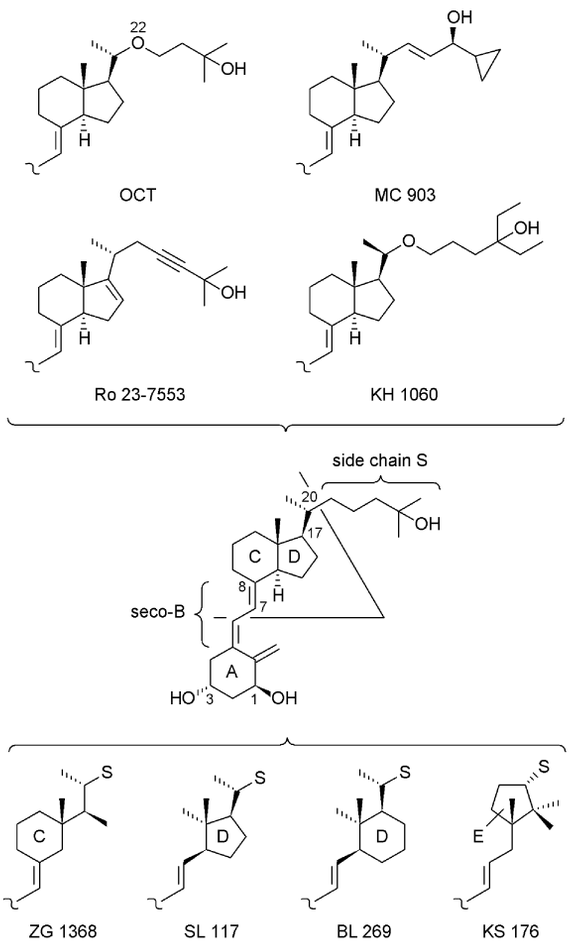
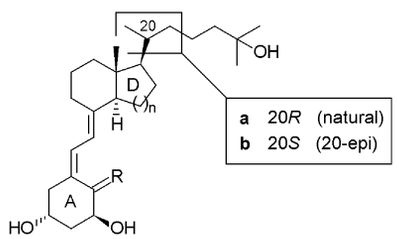


Chart 1 Selected set of examples of modifications in the side chain (top) and in the central CD-ring system (bottom) of 1,25-D_3.

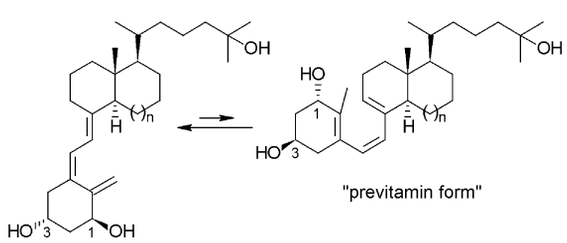
structural modifications have been introduced to each of these three parts that successfully led to derivatives possessing the desired separation of activities, the side chain has been the favourite location for analog development.[6] Moreover molecular modeling studies have indicated that there exists a link between the biological activities and the preferred spatial orientation of the side chain.[12] Seminal in this respect was the finding that 20-*epi*-1,25-D_3 is several orders of magnitude more potent than the natural hormone both in prodifferentiating and calcemic activity.[13] Another interesting modification, now in the A-ring of the molecule, consists in the deletion of C-19. Indeed, it was reported by DeLuca that 19-*nor*-1,25-D_3 possesses a biological profile with the same cell differentiative and antiproliferative activity as the natural hormone, yet with a 10-fold reduction in calcemic activity.[14] For one decade now our laboratories in Ghent and Leuven have focussed on structural modifications in the synthetically less accessible CD-ring part of the molecule.[15,16] The search has led to the development of different series of non-steroidal analogs featuring, instead of the natural *trans*-fused hydrindane‡ bicyclic system, a mono-cyclic central moiety such as a six-membered C- or a five-membered D-ring, an unnatural ringsize such as a six-membered D-ring, even an extraneous ring such as a five-membered E-ring (Chart 1).[17–20] In each of these series, derivatives were found showing a clear dissociation in activities. For the purpose of illustration, the biological activity of a selected set of analogs possessing structural modifications in the side chain or in the CD-ring system and that show a dissociation in activities is given in Table 1. Compared to 1,25-D_3 these analogs possess both a higher prodifferentiative and a lower calcemic activity, except KH 1060 and KS 176, which have a higher calcemic and a somewhat lower prodifferentiative activity, respectively.

In the same context we wish to describe here the synthesis and biological activity of a series of *trans*-decalin§ CD-ring modified 1,25-D_3 analogs in combination with classical modifications such as epimerisation at C-20 and removal of C-19 (Chart 2).[21] In addition, the isomerisation between vitamin and previtamin forms is also investigated (Scheme 2). Indeed, the



		D-ring	
		n = 1 (natural)	n = 2 (*trans*-decalin)
A-ring	**t** R = CH_2 (nat)	**1t**	**2t**
	r R = H,H (nor)	**1r**	**2r**

Chart 2 Symbolic designation used for the stereochemistry at C-20 and for the nature of the A-ring.



1t	n = 0 (natural)	**3t**
2t	n = 1 (*trans*-decalin)	**4t**

Scheme 2 Vitamin–previtamin equilibrium.

composition of this equilibrium is known to be determined by the nature of the CD-ring system.[22] In comparison with the equilibrating natural forms, it was expected that in the *trans*-decalin homologous series the corresponding previtamin derivative would be less prone to undergo a [1,7]-sigmatropic rearrangement, hence representing a system in which the biological profile of a genuine previtamin form could be assessed.

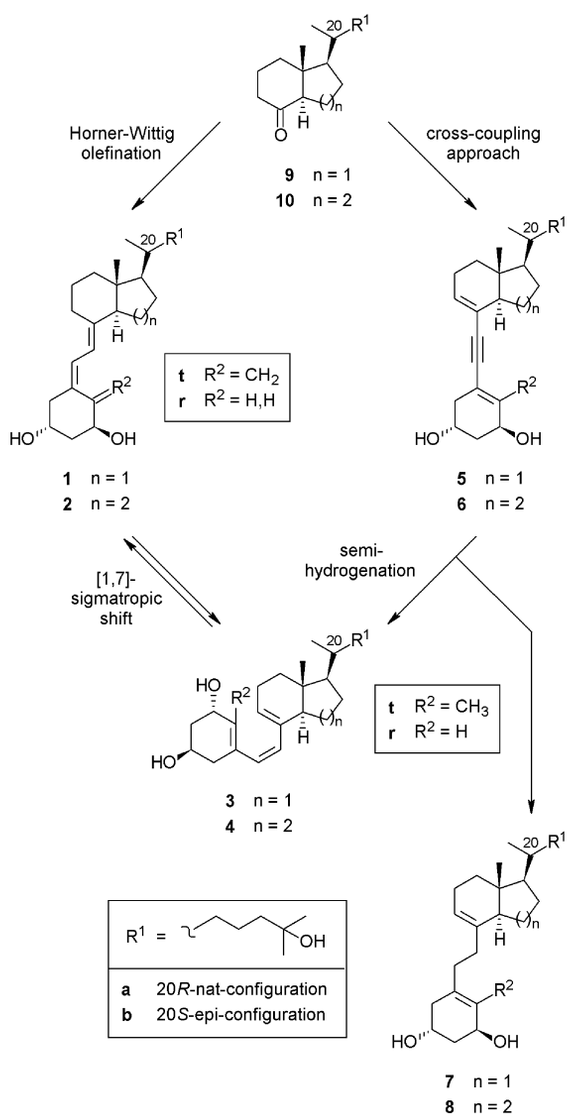
In what follows 16 different compounds corresponding to structures **1**–**8** will be compared (Scheme 3). They represent structures not only in the vitamin (**1**, **2**), and the previtamin (**3**, **4**) series, but also analogs (**5**, **6**, **7**, **8**) that were obtained in the context of the synthesis of the previtamin derivatives. In

‡ The IUPAC name for hydrindane is hexahydroindane.
§ The IUPAC name for decalin is decahydronapthalene.

Table 1 Biological activities of selected known 1,25-D_3 analogs shown in Chart 1 [a]

Code	Binding affinity		Cell differentiation and proliferation			Calcemic effect
	VDR (Pig)	DBP (Human)	HL-60	MCF-7	Keratinocytes	Serum (Mice)
OCT [b]	20	0	200	100	300	0.3
MC 903 [c]	85	7	150	—	200	1
Ro 23–7553 [d]	85	0	1000	2500	8000	8
KH 1060 [e]	45	0	4000	17000	30000	500
ZG 1368 [f]	60	20	1000	6000	6000	50
SL 117 [g]	80	10	85	85	90	0.3
BL 269 [h]	125	80	90	300	300	4
KS 176 [i]	10	19	20	30	10	<0.1

[a] All data resulted from evaluation in the Legendo laboratory at the KULeuven. The activities are presented as relative values, the reference value of 1,25-D_3 (**1at**) being defined as 100. Further details about the methodology are given in the Experimental Section. [b] 22-oxa-1,25-D_3, ref. 52. [c] 22-ene-26,27-dehydro-1,24(*S*)-D_3, ref. 53. [d] 16-ene-23-yne-1,25-D_3, ref. 54. [e] 20-*epi*-22-oxa-24,26,27-trihomo-1,25-D_3, ref. 13 and 55. [f] Ref. 18. [g] Ref. 19. [h] Ref. 20*a*. [i] Ref. 17.



Scheme 3 General synthetic strategy.

order to increase the readability of the paper the following symbols are used for structural identification: **a** and **b** refer to the natural 20*R*- and epimeric 20*S*-configuration, respectively; **t** and **r** to the natural and 19-*nor* A-ring skeleton, respectively; the odd Arabic numerals **1**, **3**, **5** and **7** refer to products possessing the natural ring system with a five-membered D-ring, and the even numerals **2**, **4**, **6** and **8** to the *trans*-decalin analogs with a six-membered D-ring.[23]

Results and discussion

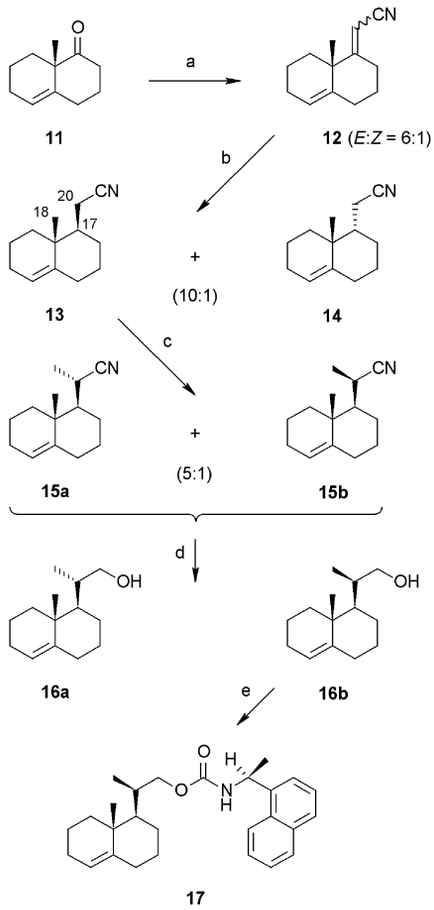
Synthesis

The general strategy for the synthesis of the discussed compounds is outlined in Scheme 3.[24,25] Central in the approach is the side-chain hydroxylated Grundmann's ketone **9** or the corresponding homologous decalone¶ **10**. Whereas the Horner–Wittig olefination, originally introduced by Lythgoe,[26] leads directly to the vitamins **1** and **2** (both in the natural and in the 19-*nor* series),[27,28] the synthesis of the corresponding previtamins **3** and **4** involves the semi-hydrogenation of the ynedienes **5** and **6**, respectively.[29] The latter are obtained now by cross-coupling of the enoltriflates derived from **9** and **10**, with a terminal alkynyl substituted A-ring.[30] Finally, derivatives **7** and **8** have been obtained as products of overreduction.

The synthesis of decalones **10a** and **10b** requires a multi-step sequence that is worked out in Schemes 4 and 5. Reaction of the known enantiomerically pure unsaturated decalone **11**[31] with the anion derived from diethyl cyanomethylphosphonate[32] gave the conjugated nitrile **12** as an unseparable mixture of *E*- and *Z*-isomers (ratio 6 : 1, respectively).[21] Subsequent selective reduction of the conjugated double bond with magnesium in methanol[33] afforded a mixture of the diastereomeric nitriles **13** and **14** (ratio 10 : 1), which were separated by flash chromatography.[21] The assignment of the major isomer to **13**, in which the cyanomethyl substituent at C-17 occupies the expected more stable equatorial position,[34] rests on the following NOE experiment: irradiation of the C-18 methyl group (δ 0.96) showed a 4.2% enhancement of H-20 (δ 2.49) in the case of the major isomer **13**, whereas in the case of the minor isomer **14** no such enhancement was observed. Our previously reported synthetic sequence towards *trans*-decalin analogs **2at** and **2bt** involved alkylation of the enolate derived from cyanomethyl derivative **13** with the complete C_{22}–C_{27} side chain segment, followed by conversion of the cyano function into the C-21 methyl group.[21] The configuration at C-20 in the major isomer was found to correspond to the natural (20*R*)-configuration.[35] In analogy with this result the alkylation of **13** with iodomethane leads to a mixture of **15a** and **15b** in which **15a** predominates (ratio 5 : 1). This assigment was confirmed by the ^{1}H NMR spectral data: comparison of the observed and calculated vicinal coupling constant values for $J(H_{17},H_{20})$ (**15a**: obsd <1.0 Hz, calcd 1.0 Hz; **15b**: obsd 3.1 Hz, calcd 4.0 Hz) indicates **15a**, in which C-20 possesses the natural configuration, to be the major stereoisomer.[36]

After conversion of the nitrile function into the corresponding primary alcohol (diisobutylaluminium hydride, 2 times), the two diastereomers **16a** and **16b** were separated by chromato-

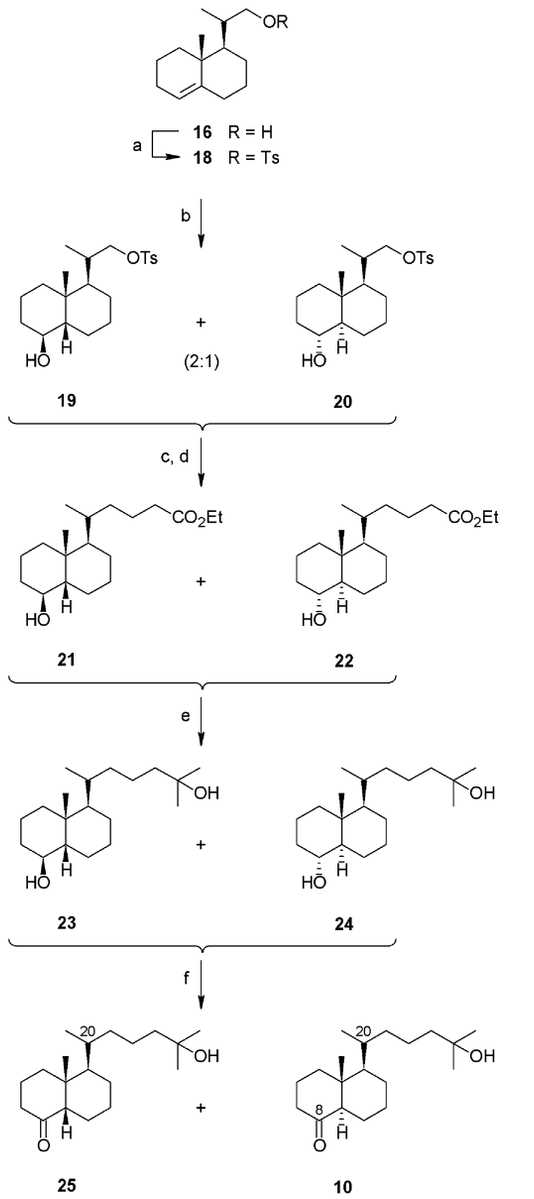
¶ The IUPAC name for decalone is octahydronapthalenone. The IUPAC name for triflate is trifluoromethanesulfonate.



Scheme 4 (a) $(EtO)_2P(O)CH_2CN$, $NaNH_2$, THF, rt, 6 h; **11**, THF, rt, 12 h (96%); (b) Mg, MeOH, rt, 18 h (92%); (c) *n*-BuLi, *i*-Pr_2NH, THF, −20 °C, 30 min; **13**, THF, −78 °C, 1 h; MeI, THF, −78 ⟶ −20 °C, 3 h (93%); (d) DIBAL-H, *n*-hexane–Et_2O, −78 ⟶ 0 °C, 4 h (2 times) (64%); (e) (*S*)-(+)-$C_{10}H_7CH(CH_3)NCO$, $CF_3SO_3Si(CH_3)_3$, CH_2Cl_2, rt, 40 min (97%).

graphy. In view of the applied reaction conditions for transforming the mixture of nitriles **15a** and **15b** into the corresponding alcohols **16a** and **16b**, substantial isomerisation was not expected. Hence the major isomer that was obtained after reduction of the nitrile function was anticipated to correspond to **16a**, possessing the "natural" configuration at C-20. A further indication that this assignment is correct follows again from the ^{1}H NMR spectral data and from the result of molecular modeling. Fig. 1 shows the predominant orientation of the side chain as calculated for **16a** and **16b**. This is in accordance with (i) the large shift difference of 0.52 ppm between the two hydrogen atoms on C-22 in **16a** as compared to **16b** (0.02 ppm); (ii) the NOE enhancement (3.8%) of the signal of the low-field hydrogen atom on C-22 (δ 3.81) upon irradiation of the C-18 methyl group (δ 1.06), which is only observed in the case of **16a**, and (iii) the experimental vicinal coupling constants of H-20 with the hydrogens on C-22 (**16a**: obsd 3.5, 9.9 Hz, calcd 2.9, 9.6 Hz; **16b**: obsd 6.8, 7.5 Hz, calcd 6.1, 6.5 Hz). The structural assignment within the b-series also follows from the X-ray diffraction analysis of crystalline **17** (Fig. 2).[37] The absolute structure could not be determined from the X-ray data, but was established from the synthesis pathway. Indeed, the latter carbamate was obtained by reaction of **16b** with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate in the presence of trimethylsilyl triflate. Obviously, both the expected relative configuration at C-13, C-17 and C-20, and the absolute configuration as shown in **16** is thereby unambiguously proven.

The further conversion of unsaturated alcohols **16a** and **16b** into the corresponding decalones **10a** and **10b**, respectively, is outlined in Scheme 5 (the same scheme is used for the two



a C-20 stereochemistry corresponding to 20*R*-natural configuration
b C-20 stereochemistry corresponding to 20*S*-epi configuration

Scheme 5 (a) TsCl, pyridine, 0 °C, 12 h (**18a**: 92%; **18b**: 91%); (b) BH_3·THF, THF, −20 °C, 12 h; 2 M NaOH, 35% H_2O_2, −30 °C ⟶ rt, 30 min (**19a** + **20a**: 82%; **19b** + **20b**: 82%); (c) NaI, acetone, 60 °C, 12 h; (d) $NiCl_2$, Zn, CH_2=$CHCO_2Et$, pyridine, 65 °C, 45 min; mixture of iodides from (c), pyridine, rt, 1.5 h (**21a** + **22a**: 80%; **21b** + **22b**: 72%); (e) MeMgBr, THF, −5 °C ⟶ rt, 1 h (**23a** + **24a**: 93%; **23b** + **24b**: 91%); (f) PDC, CH_2Cl_2, rt, 12 h (**25a** + **10a**: 88%; **25b** + **10b**: 86%).

configurational series). The sequence involves two basic operations consisting of the establishment of a *trans*-fused C-8 decalone and the introduction of the C-25 hydroxylated side chain. The oxidative hydroboration procedure applied to tosylates **18a** and **18b**, obtained in the classical way from the corresponding alcohols **16a** and **16b**, respectively, led in both cases to a 2 : 1 mixture of *cis*- and *trans*-fused isomers (**19a** and **20a** from **18a**, and **19b** and **20b** from **18b**, respectively). On mere steric grounds the preferred addition to the convex face of the bicyclic molecule resulting in the preferred formation of the *cis*-fused isomer (*i.e.*, **19a** and **19b**) was anticipated.[38] Indeed, the major isomer in the obtained 2 : 1 mixture is the *cis*-fused isomer in which the C-18 methyl group in the ^{1}H NMR resonates at lower field. The same trend is also observed when comparing subsequent *cis*- and *trans*-fused isomers; the average

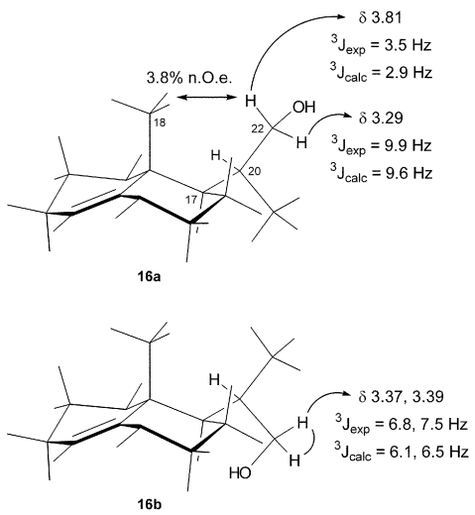


Fig. 1 Predominant orientation of the side chain as calculated for **16a** and **16b**, and the experimental and calculated ^{1}H NMR vicinal coupling constants *J* for the hydrogens on C-22.

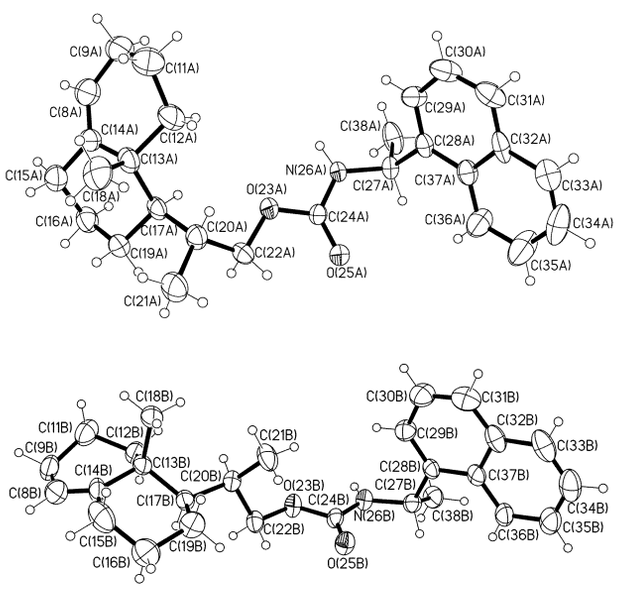
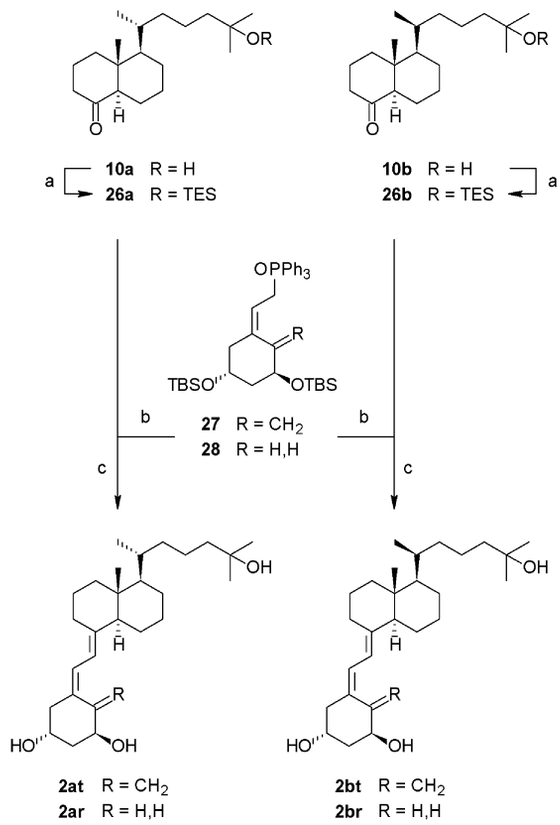


Fig. 2 Perspective view of the crystal structure of two (molecules A and B) of the four independent molecules in the unit cell of crystalline **17**, with the adopted atom numbering.

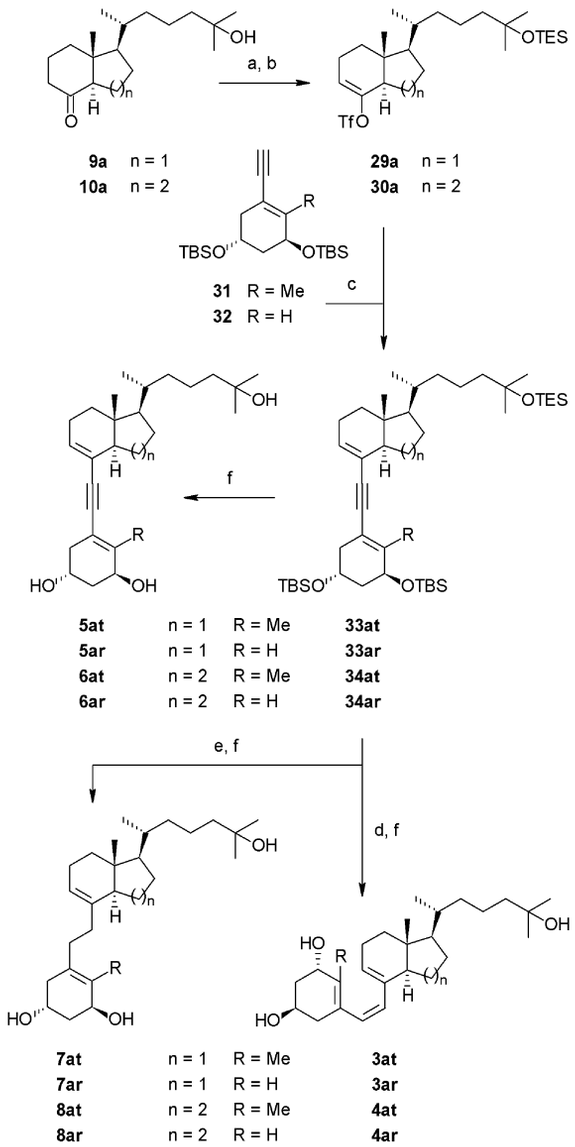
shift value for **10**, **20**, **22**, and **24**: 0.70–0.80 ppm, and for **19**, **21**, **23**, and **25**: 0.85–1.13 ppm.[39] For the introduction of the side chain we used an improved procedure developed by Mouriño which involves a zinc–copper mediated conjugate addition of an iodide (obtained from the corresponding tosylates **19** and **20** *via* Finkelstein's protocol) to ethyl acrylate under aqueous sonochemical conditions (85% yield).[40,41] The mixture of esters **21a** and **22a** (and of **21b** and **22b** in the epimeric series) was not separated but treated successively with methylmagnesium bromide and pyridinium dichromate. At this stage the *cis*- and *trans*-fused decalones **25a** and **10a** (and **25b** and **10b** in the 20-epimeric series) were separated and spectroscopically identified. In particular the resonance of the angular methyl group has a diagnostic value: a characteristic high field resonance is observed for the *trans*-fused derivatives (δ 0.74 for **10a** and 0.75 for **10b**) when compared to the corresponding *cis*-fused compounds (δ 1.07 for **25a** and 1.13 for **25b**).[39] Conversion of the *cis*-fused decalones into the more stable desired *trans*-fused isomers was readily realised by treatment in base.[42]

The preparation of the vitamin analogs **2at**, **2ar** (and **2bt**, **2br**) proceeded *via* the classical Lythgoe coupling of the triethylsilyl protected decalone **26a** (and **26b**).[43] This very reliable and stereoselective Wittig–Horner type olefination involves reaction with the A-ring phosphine oxide **27** (natural A-ring)[44] and **28** (19-nor A-ring),[28,45] followed by deprotection of the different trialkylsilyl ether groups (Scheme 6).



Scheme 6 (a) TESCl, imidazole, CH_2Cl_2, rt, 3 h (**26a**: 88%; **26b**: 84%); (b) **27** (**28**), *n*-BuLi, THF, −78 °C, 1 h; **26a** (**26b**), THF, −78 °C ⟶ rt, 2 h; (c) TBAF, THF, rt, 12 h.

The synthesis of the previtamin derivatives **3** and **4** is highlighted in Scheme 7. The chosen strategy involves coupling of vinyl triflates **29a** and **30a** with the A-ring terminal alkynes **31** (natural A-ring)[46] and **32** (19-nor A-ring)[47] following the procedure developed by Castedo and Mouriño and co-workers.[48] The sequences leading to the previtamin derivatives in the natural hydrindane series have been described before, both for the preparation of the 19-natural (**3at**; pre-D_3)[9] and the 19-nor (**3ar**)[47] derivative. The required hydrindanone intermediate **9a** was obtained following the known procedure involving ozonolysis of vitamin D_3, followed by direct hydroxylation at the 25-position.[49] The conversion of both ketones **9a** and **10a** into the reactive vinyl triflate intermediates **29a** and **30a** proceeded *via* prior protection (triethylsilyl chloride, dimethylaminopyridine, dichloromethane), and treatment of the enolate anions (LDA, THF) with *N*-phenyltriflimide in THF (>90% yield).[50] The subsequent coupling reactions followed by classical deprotection led to the four yne–diene type analogs **5at**, **5ar** (natural CD-series), and **6at**, **6ar** (*trans*-decalin series). For the synthesis of the previtamin derivatives **3** and **4**, the coupling process is immediately followed by the semi-hydrogenation of the C-6,C-7 yne-moiety with Lindlar catalyst in the presence of quinoline. Careful reaction control is required since overreduction to yield the corresponding saturated derivatives at C-6,C-7, **7** and **8** readily occurs. In the context of analog development, however, this side reaction was gratefully acknowledged as a novel series of analogs became available for biological screening. Due to the known facile conversion of the natural previtamin D_3 (**3at**) to the corresponding vitamin form (**1at**, calcitriol),[9] the semi-hydrogenation of compound **33at** was not undertaken in this work.



Scheme 7 (a) TESCl, DMAP or imidazole, CH_2Cl_2, rt, 3–12 h (from **9a**: 96%; from **10a**: 88%); (b) *n*-BuLi, *i*-Pr_2NH, THF, 0 °C, 20 min; ketone–silyl ether from (a), THF, −78 °C, 45 min; then ⟶ rt over 2 h; $(CF_3SO_2)_2NC_6H_5$, THF, −78 °C, 10 min; then ⟶ rt over 4 h (**29a**: 88%; **30a**: 71%); (c) **31** (**32**), CuI, $Pd(PPh_3)_2(OAc)_2$, Et_2NH, MeCN, rt, 1 h (**33at**: 86%; **34at**: 92%; **34ar**: 91%); (d) H_2, Lindlar catalyst, quinoline, EtOAc, rt (protected **3ar**: 86%; **4at**: 54% from **6at**); (e) H_2, Lindlar catalyst, EtOAc, rt (**8at**: 34% from **6at** under conditions (d)); (f) TBAF, THF, rt, 12 h (**3ar**: 80% from **33ar**; **4ar**: 83% from **34ar**; **5at**: 90%; **5ar**: 92% from **29a**; **6at**: 91%; **6ar**: 96%; **7at**: 66% from **33at**; **7ar**: 81% from protected **3ar**; **8ar**: 81% from **34ar**).

Biological evaluation

The biological evaluation of the parent compound (**1at**) and the different analogs has included the determination of (1) the affinity for the pig intestinal mucosa vitamin D receptor (VDR) and for the human vitamin D binding protein (hDBP), (2) the prodifferentiating activity, tested on promyelocytic leukemia HL60 cells, (3) the *in vitro* antiproliferative effects, determined on breast cancer MCF-7 cells, and (4) the *in vivo* calcemic activity, tested in vitamin D-replete NMRI mice. Results are shown in Tables 2–3.

Discussion

The search for analogs showing a separation in prodifferentiating and calcemic activities followed the 1981 report by Suda and his associates who indicated for the first time that 1,25-D_3 inhibits the proliferation of myeloid leukemic cells and induces their differentiation into monocytes–macrophages.[51] After the 1981 report, analogs were developed on the basis of the idea that by modifying the chemical structure of 1,25-D_3 one could separate the differentiation inducing activities from the calcium regulating activities. As mentioned earlier the side-chain portion has been the preferred location for introducing structural modifications in the development of vitamin D analogs. The first analog in which the desired separation of activities was reported is 22-oxa-1α,25$(OH)_2$–D_3 (OCT).[52] Other important analogs in this respect are shown in Chart 1 and include calcipotriol (MC 903),[53] 16-ene-23-yne-1α,25$(OH)_2$–D_3 (Ro 23–7553)[54] and 20-*epi*-22-oxa-24,26,27-trihomo-1α,25$(OH)_2$–D_3 (KH 1060).[13,55] The latter compound exhibits a 20,000 fold increased activity on cell differentiation and immune responses but with increased calcemic activity. Its structure accumulates several side-chain modifications that have been reported to enhance the cell-differentiation activity: 20-epimerisation, 22-oxygen insertion, and side-chain elongation.[6] Interesting modifications in the A-ring include 19-nor-1α,25$(OH)_2$–D_3,[14] 1α-(hydroxymethyl)-25-OH-D_3,[56] 2β-(3-hydroxypropoxy)-1α,25-(OH)-D_3 (ED-71)[57] and 2-methyl alkylated derivatives.[58] The 19-nor modification in particular is crucial in the context of vitamin–previtamin equilibration (*vide infra*). Analogs that have been developed in the period 1985–1995 have been reviewed in a comprehensive manner by Bouillon, Okamura and Norman: 280 analogs were listed involving in total 820 structural variations of 1,25-D_3.[6] Following this review many more analogs have been described, in particular also novel non-steroidal compounds.[59,15]

The 20-*epi*-modification

In the context of the study of structure–function relationships the 20-epimerisation modification has taken a unique position. Following the 1991 report by Binderup about the increased overall activity of 20-*epi*-1,25-D_3 it became clear that the orientation in space of the side chain plays an important role.[13] Okamura in Riverside developed a dot-map approach in order to visualise the portions in space that were accessible for the side-chain, with focus on the 25-OH group.[60] This approach was further used by Yamada in a comprehensive structure–function relationship study.[61] In particular, it was found that analogs with preferred side-chain orientation in the region represented as EA in Fig. 3 consistently possess a higher biological activity (except the affinity for DBP).[12] We developed a similar procedure for defining a volume in space, the preferred occupation of which would correspond to high prodifferentiating (HL-60) and antiproliferative (MCF-7) activity.[62] This "active" volume is represented by a red sphere and coincides with the above mentioned EA region.

Inspired by the observation that severe structural modifications in the central CD-ring part are coupled with a reduction in calcemic activity (Table 1), we became interested in the development of analogs in which the applied modifications would direct the side chain in the presumed active region. This has previously led to the development of 6D-analogs, characterised by a six-membered 1,3-dimethyl substituted (C-16,C-14) cyclohexane D-ring with reduced conformational mobility of the side chain.[20b] Fig. 3 shows the result of the conformational analysis procedure (see Experimental Section) for the side-chain orientation of **1a**, **1b**, **2a** and **2b**. Inspection of the figure learns that substantial occupation of the active volume is realised by **2a**, but not by the 20-*epi* analog **2b**.[63] This is particularly well reflected in the results of the biological evaluation: the cell differentiative, antiproliferative *and* calcemic activities of **2at** are substantially higher than of **2bt**, whereas the affinity for the VDR is almost identical. The same trend, although somewhat less pronounced, is also observed for the corresponding 19-*nor* derivatives **2ar** and **2br**. This *trans*-decalin pair represents one of the rare examples where the 20-epimer shows a reduced

Table 2 Biological activities of 1,25-D_3 analogs **1**–**2**[a]

Code	Binding affinity		Cell differentiation and proliferation			Calcemic effect
	VDR (Pig)	DBP (Human)	HL-60	MCF-7	Kerat.[b]	Serum (Mice)
1,25-D_3 (**1at**)	100	100	100	100	100	100
20-*epi*-1,25-D_3 (**1bt**)	90	0.2	3000	5000	2200	800
19-*nor*-1,25-D_3 (**1ar**)	90	40	150	90	—	2
19-*nor*-20-*epi*- (**1br**)[c]	—	—	—	—	—	—
CY 10012 (**2at**)	90	7	1000	2250	1400	200
CY 943 (**2bt**)	80	1	70	85	70	<0.1
CY 10010 (**2ar**)	75	2	1000	1000	1000	100
CY 941 (**2br**)	8	0	10	30	30	<0.1

[a] All data resulted from evaluation in the Legendo laboratory at the KULeuven. The activities are presented as relative values, the reference value of 1,25-D_3 (**1at**) being defined as 100. Further details about the methodology are given in the Experimental Section. [b] Keratinocytes. [c] The corresponding activities of this analog have not yet been described in the current literature.

Table 3 Biological activities of 1,25-D_3 analogs **3**–**8**[a]

Code	Binding affinity		Cell differentiation and proliferation		Calcemic effect
	VDR (Pig)	DBP (Human)	HL-60	MCF-7	Serum (Mice)
Pre-1,25-D_3 (**3at**)[b]	4	7	<5	—	—
GAO 306 (**3ar**)	5	9	8	30	<0.25
IM 9053 (**4at**)	0.5	7	700	2000	20
GAO 182 (**4ar**)	8	4	85	350	2
GAO 315 (**5at**)	9	60	7	30	—
GAO 205 (**5ar**)	50	20	9	20	<0.1
IM 902 (**6at**)	85	80	100	100	10
GAO 183 (**6ar**)	80	20	300	2300	10
GAO 320 (**7at**)	3	75	4	30	—
GAO 305 (**7ar**)	10	30	8	30	—
IM 9102 (**8at**)	70	20	85	100	0.5
GAO 181 (**8ar**)	100	30	600	2200	10

[a] All data resulted from evaluation in the Legendo laboratory at the KULeuven. The activities are presented as relative values, the reference value of 1,25-D_3 (**1at**) being defined as 100. Further details about the methodology are given in the Experimental Section. [b] Pentadeuterio d_5 derivative, ref. 66.

biological activity when compared to the derivative with the natural configuration. In spite of the usefulness of the active volume concept as illustrated above with **2a** and **2b**, it should be stressed that it does not provide a solution for the discovery of analogs with dissociation in activity since virtually without exception the more potent derivatives in cell differentiation and antiproliferation also possess a higher calcemic activity.

The behavior of the classical 20-*epi* analogs, such as 20-*epi*-1,25-D_3 (**1bt**, MC 1288) and KH 1060, is controversial. Indeed, whereas the nuclear receptor activation mechanism somehow implies that the affinity of the ligand for the receptor is proportional to the activation, in the case of these 20-*epi* analogs the affinity for the n-VDR is similar or even lower than that of 1,25-D_3, whereas their transcriptional activity is several orders of magnitude higher. The molecular origin of this behavior is still under discussion.[64,65]

The previtamin form

Pre-1,25-D_3 (**3at**, as a pentadeuterio derivative) has been shown to stimulate nongenomic but not genomic biological responses.[66] Since the rapid nongenomic responses have been attributed to the interaction of 1,25-D_3 and a putative membrane receptor protein,[67] the interesting hypothesis was raised that different conformations of 1,25-D_3, readily accessible through rotation about the C-6,C-7 bond, would be involved in binding to the nuclear and membrane receptor proteins.[68] Whereas the more stable extended transoid geometry has been shown experimentally to be involved in the binding in the LBD of the n-VDR,[11] the compact steroid-like cisoid geometry would be involved in the binding to the m-VDR.[69] Studies involving the biological evaluation of analogs locked into the s-*cis* geometry have substantiated this model.[70]

Pre-1,25-D_3 (**3at**) is unique since it can be considered as a tautomer of 1,25-D_3 in the cisoid conformation.[71] Whereas the direct screening of the natural previtamin form is problematic, due to its propensity (even though slow) to convert into the active vitamin form, this problem was circumvented in an elegant way by Okamura and co-workers through the study of the kinetically more stable 9,14,19,19,19-pentadeuterio previtamin derivative.[9,66] An alternative but more drastic solution resides in the study of the 19-*nor* derivative (**3ar**), which obviously is unable to isomerise to the vitamin form.[72] Both studies have shown that as far as the transcriptional activity is concerned the previtamin form has lost most of its biological activity *in vitro* and *in vivo*. In the same context the previtamin form of the *trans*-decalin analog (**4at**) is an attractive derivative to study. Indeed, whereas in the natural hydrindane series the previtamin–vitamin equilibrium composition between **1at** and **3at** is largely in favor of the vitamin derivative (90 : 10), in the case of the *trans*-decalin analogs this is less pronounced, *i.e.* the ratio of **2at** : **4at** equals 75 : 25 and the latter equilibrium is very slowly installed (5 h at 85°C in CD_3CN). In both cases subtle conformational preferences related to the unfavourable position of the previtamin Δ^8 bond in the *trans*-fused system lead to the preferred formation of the vitamin form,[73] but this is less pronounced in the *trans*-decalin case. The results of the biological screening were quite unexpected since they showed **4at** to possess a remarkably high prodifferentiating (HL-60) and antiproliferative (MCF-7) activity, with no affinity for the n-VDR; the calcemic activity was moderately reduced. In order to rule out any possible prior conversion to the vitamin form, the

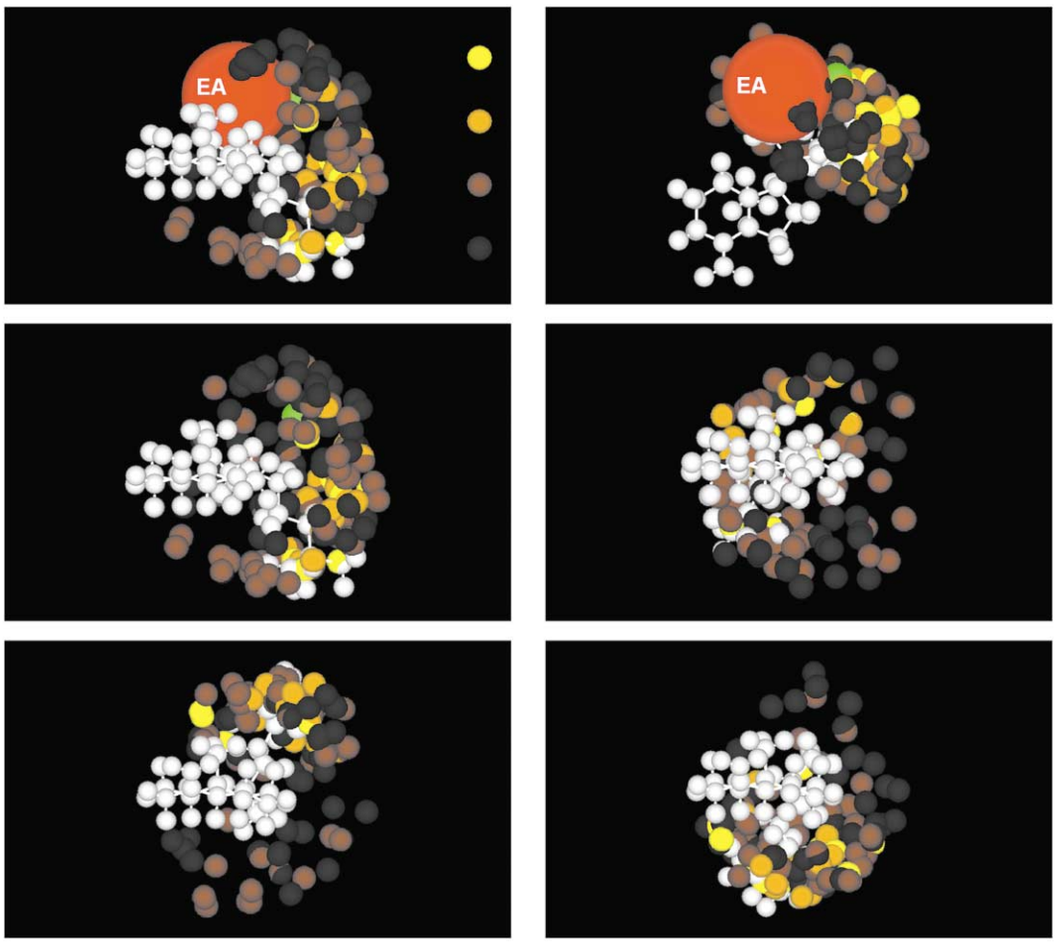


Fig. 3 Volume maps representing the conformational behavior of the side chain of 1,25-D_3 (**1a**), 20-*epi*-1,25-D_3 (**1b**), and *trans*-decalin analogues **2a** and **2b** (truncated model); colored spheres indicate positions of O-25: yellow to brown represent conformations within 0–5, 5–10, 10–15, and 15–20 kJ mol^{-1}, respectively, of the minimum energy form (grey). Top: 1,25-D_3 (**1a**); front view (left); top view (right); indicated EA region following ref. 61; active volume (red) according to ref. 62; position of O-25 in receptor-bound conformation (green) taken from ref. 11. Middle: front view of 1,25-D_3 (**1a**) (left) and 20-*epi*-1,25-D_3 (**1b**) (right). Bottom: front view of *trans*-decalin analogues **2a** (left) and **2b** (right).

corresponding 19-*nor*-previtamin **4ar** was also synthesised and its biological activity assessed. This further modification results in an approximate 10-fold reduction in activity compared to **4at** (HL-60, MCF-7, and calcemic effects), but **4ar** still possesses a substantial prodifferentiative and antiproliferative activity. Does this unexpected result originate from a genuine previtamin triene effect or rather from a *trans*-decalin effect? In order to settle this unambiguously **3ar** was resynthesised and fully evaluated.[74] Comparison of the results obtained for **3ar** and **4ar** confirm that the *trans*-decalin structure is responsible.

The seco-B-ring modification

The unexpected biological activity of a previtamin derivative prompted us to also screen the yne–diene derivatives **6** that were originally synthesised as precursor molecules for the previtamin derivatives.[75] As the hydrogenation procedure easily leads to full reduction of the C-6,C-7 triple bond, the corresponding saturated analogs **8** were also prepared and screened. Again the results were unexpected. Both **6at** and **8at** possess an activity quite similar to 1,25-D_3, except for a reduced calcemic activity. The corresponding 19-nor derivatives **6ar** and **8ar** have an almost identical profile with, however, a much more pronounced prodifferentiative (HL-60) and antiproliferative activity (MCF-7) compared to **6at** and **8at**. Again, the origin of this behaviour was examined through the evaluation of the corresponding natural CD-ring derivatives, *i.e.* **5at**, **5ar**, **7at**, and **7ar**. These compounds, however, have only a marginal vitamin D like activity profile and are 10 to 100 times less potent than their counterparts with the *trans*-fused decalin system. Hence again the origin of the biological behaviour may be attributed to the particularity of the *trans*-decalin system.

In conclusion, several analogs have been developed, characterised by a *trans*-decalin modified central CD-ring system, which show surprising biological activity when compared to the corresponding derivatives with a natural hydrindane structure. It is expected that these derivatives will be useful in the discovery of the detailed molecular mechanism of the transcriptional activity of 1,25-D_3 and related compounds.

Experimental

Detailed information about the preparation of all derivatives, except a few general procedures of key experiments, is deposited as Electronic Supplementary Information (ESI).

Coupling reaction of ketones 26a and 26b with phosphine oxides 27 and 28 – general procedure

To a solution of A-ring phosphine oxide **27** (**28**) (0.67 mmol) in dry THF (7.5 cm^3) was added dropwise *n*-BuLi (2.5 M solution in hexanes, 0.24 cm^3, 0.60 mmol) at −78 °C under Ar. The formed dark red solution was stirred at −78 °C for 1 h and a solution of ketone **26a** (**26b**) (0.17 mmol) in dry THF (2.5 cm^3) was added dropwise. The red solution was stirred at −78 °C for 2 h and was then allowed to warm to rt. The reaction mixture was loaded onto a silica gel column, the reaction product was eluted (*n*-hexane–EtOAc, 5 : 1) and further purified by HPLC to give the protected 1,25-D_3 analog.

Desilylation of the protected 1,25-D_3 analogs using TBAF – general procedure

To a solution of the protected 1,25-D_3 analog (0.14 mmol) in THF (4 cm^3) was added tetrabutylammonium fluoride (TBAF, 1 M solution in THF, 2.1 cm^3, 2.1 mmol). The reaction mixture was stirred at rt for 12 h and then loaded onto a silica gel column. The reaction product was eluted (*n*-pentane–acetone, 1 : 1) and further purified by HPLC to give the 1,25-D_3 analog.

Coupling reaction of enol triflates 29a and 30a with A-ring intermediates 31 and 32 – general procedure

To a solution of enol triflate **29a** (**30a**) (0.5 mmol) and A-ring synthon **31** (**32**) (0.5 mmol) in a mixture of Et_2NH (2.5 cm^3) and CH_3CN (5 cm^3) were added CuI (10 mg, 0.05 mmol) and bis(triphenylphosphine)palladium(II) acetate ($Pd(PPh_3)_2(OAc)_2$, 37.5 mg, 0.05 mmol). The reaction mixture was stirred at rt for 1 h and diluted with *n*-pentane–EtOAc 99 : 1 (25 cm^3). The solution was passed through a short pad of silica gel, which was rinsed with *n*-pentane–EtOAc 99 : 1 (250 cm^3). Concentration under reduced pressure left a residue, which was purified by HPLC to give the protected 1,25-D_3 analog.

Semi-hydrogenation and hydrogenation of the yne–diene type derivatives – general procedure

A mixture of the ynediene **33** or **34** (0.029 mmol), Lindlar catalyst (0.02 g), and quinoline (2% solution in *n*-pentane, 0.1 cm^3) in EtOAc (2 cm^3) was stirred under a H_2 atmosphere at rt. The reaction was carefully monitored by TLC for disappearance of the starting material. The reaction mixture was then passed through a pad of silica gel and concentrated under reduced pressure to yield the semi-hydrogenated derivative. In the absence of quinoline in the reaction mixture the procedure gave the corresponding derivative with a fully hydrogenated triple bond.

Molecular modelling and conformational analysis

All modelling was performed by molecular mechanics calculations using the MacroModel V6.0 molecular modelling programme of Still[36] run on a Silicon Graphics Octane workstation and using the programme's standard parameters. Conformational analysis of the side chain of compounds **1a**, **1b**, **2a** and **2b** was carried out on model compounds in which the A-ring and the diene system up to C6 were replaced by a H atom. Rotations with 60° increments were applied to the rotatable C–C bonds of the side chain, while the 25-OH was rotated with increments of 120°. The so generated 23,328 starting conformations were minimised using the MM2* force field implementation of the programme and the conformations within 20 kJ mol^{-1} of the minimum energy form were retained. All conformations of each compound were then overlaid using C-13 as common origin (x, y, $z = 0$), C-14 was positioned in the yz-plane ($x = 0$) and the position of C-18 was made to coincide with the positive y-axis (x, $z = 0$). A ball-and-stick model was generated of the minimum energy conformation and the position of O-25 in each of the local energy minima within the given energy window was represented by a yellow–brown sphere to obtain the views shown in Fig. 3. The procedure for the calculation of the active volume shown as a red sphere has been reported earlier.[62] The position of O-25 of 1,25-D_3 as bound to the n-VDR and shown as a green sphere was taken from the work of Moras *et al*.[11]

Biological evaluation

1,25-D_3 (**1at**) was a gift from Duphar (Weesp, The Netherlands; J. P. van de Velde); OCT was a gift from Chugai Pharmaceutical Co (Japan); MC 1288 (**1bt**), MC 903, and KH 1060 were kind gifts from Leo Pharmaceuticals (Ballerup, Denmark; L. Binderup); Ro 23–7553 was a gift from Hoffmann-La Roche Inc. (USA).

Binding studies

The affinity of the decalin analogs of 1,25-D_3 to the vitamin D receptor was evaluated by their ability to compete with [^{3}H]1,25-D_3 for binding to high speed supernatant from intestinal mucosa homogenates obtained from normal pigs as described previously.[17b] The relative affinity of the analogs was calculated from their concentration needed to displace 50% of [^{3}H]1,25-D_3 from its receptor compared with the activity of 1,25-D_3. Binding to hDBP was performed at 4 °C essentially as described previously.[76]

MCF-7 proliferation assay

The human breast carcinoma (MCF-7) cell line was obtained from the American Tissue Culture Company (Rockville, MD). The antiproliferative activity on MCF-7 cells was assessed by evaluating [^{3}H]thymidine incorporation. Cells were seeded in 96-well plates (7500 cells per well) and 1 μCi [methyl-^{3}H]thymidine (ICN Biomedicals, Costa Mesa, CA) was added 72 h after the initiation of treatment. Cells were semi-automatically harvested after an additional 6 h of incubation on filter plates only retaining incorporated thymidine (GF/C Filter and Filtermate Universal Harvester, Packard Instrument, Meriden, CT). Counting was performed using a microplate scintillation counter (Topcount, Packard).

Differentiation of HL-60 cells

The human promyelocytic leukemia cell line (HL-60) was obtained from the American Tissue Culture Company (Rockville, MD). HL-60 cells were seeded at 4×10^4 cells cm^{-3} in 25 cm^2 Falcon tissue chambers using RPMI 1640 medium supplemented with 20% fetal calf serum (Sera-Lab, W. Sussex, UK) and gentamycin (50 μg cm^{-3}; Gibco, Roskilde, Denmark). 1,25-D_3, analogs or vehicle were added the day after plating. After 4 days of culture, differentiation was measured by using the NBT reduction assay as described previously.[77]

In vivo studies

NMRI mice were obtained from the Proefdierencentrum of Leuven (Belgium) and fed on a vitamin D-replete diet (0.2% calcium, 1% phosphate, 2000 U vitamin D kg^{-1}; Hope Farms, Woerden, The Netherlands). The calcemic effect of the decalin analogs was tested in NMRI mice by daily subcutaneous injection of 1,25-D_3, its analogs or the solvent during 7 consecutive days, using serum calcium concentration as a parameter.

X-Ray crystal structure analysis of compound 17[78]

Single crystals suitable for X-ray analysis were obtained by recrystallisation from *n*-pentane.

Crystal data

$C_{27}H_{35}NO_2$; M = 405.56 g mol^{-1}; monoclinic, space group $P2_1$, a = 12.368(3), b = 17.711(4), c = 21.575(4)Å, β = 92.382(4)°, U = 4722(2)Å^3, T = 293(2), Z = 8, d_{calcd} = 1.141 Mg/m^3, μ(Mo–Kα) = 0.071 mm^{-1}, 32,705 reflections collected, 13,128 unique (R_{int} = 0.032), 7,859 observed reflections [$I > 2\sigma(I)$].

Structure solution and refinement

Solution by direct methods,[79] refinement by full matrix least-squares[80] on F^2, non-H atoms with anisotropic displacement parameters, H atoms geometrically positioned and treated as riding atoms; data : parameters ratio: 13128 : 1081; R = 0.0420 (observed data), $R_w(F^2)$ = 0.1120 (all data).

Description of the structure

The crystal structure of **17** contains four independent molecules in the unit cell, which have been indicated as molecules A, B, C and D. They differ in conformation: molecules A and B (Fig. 2) have very different conformations of their side chains, while molecule C is similar to B and molecule D to A. The crystal packing is governed by rather weak intermolecular hydrogen bonds. More details are provided in Table 4 (relevant torsion angles of the side chain of **17**) and in Table 5 (geometry of the intermolecular hydrogen bonds in the crystal structure of **17**), and are found in the Electronic Supplementary Information (ESI).

Acknowledgements

Financial assistance of the "Fonds voor Wetenschappelijk Onderzoek Vlaanderen" (project G.0150.02, G.0036.00) is gratefully acknowledged.

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